Chapter 13

Genetic Manipulation of *Cryptosporidium parvum* with CRISPR/Cas9

Adam Sateriale, Mattie Pawlowic, Sumiti Vinayak, Carrie Brooks, and Boris Striepen

Abstract

Cryptosporidium parvum can be reliably genetically manipulated using CRISPR/Cas9-driven homologous repair coupled to in vivo propagation within immunodeficient mice. Recent modifications have simplified the initial protocol significantly. This chapter will guide through procedures for excystation, transfection, infection, collection, and purification of transgenic *Cryptosporidium parvum*.

Key words Transfection, Transgenics, CRISPR, Cas9, Genetics

1 Introduction

Cas9 is an RNA-guided endonuclease and part of the bacterial immunity pathway CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) [1]. When provided with an RNA guide sequence, the Cas9 protein will scan DNA for a precise match prior to cutting, allowing to deliver directed double-strand breaks in the target genome. In many eukaryotic cells, double-strand breaks in genomic DNA are quickly repaired by nonhomologous end joining; DNA is trimmed back from the break site and then re-ligated [2]. *Cryptosporidium* appears to lack the essential machinery for nonhomologous end joining; therefore, any break in the DNA must be repaired by homologous recombination [3]. This can be exploited by providing homologous DNA stretches flanking a selection cassette. The combination of a targeted cut by Cas9 with matching homologous flanks will direct recombination of the desired locus with high fidelity, and transgenic parasites can be drug selected for during an in vivo infection [4]. To enrich and propagate these transgenic *C. parvum* strains, we use immunocompromised mice (Fig. 1). We prefer IFNγ-deficient mice for creating transgenic strains as they yield the highest level of infection;

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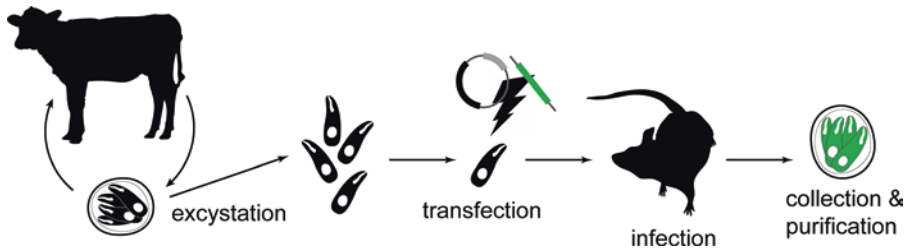

Fig. 1 Overview of *Cryptosporidium parvum* genetic manipulation. *Cryptosporidium* sporozoites are induced to excyst and then transfected by electroporation. Next, exogenous DNA inserts into the parasite genome using Cas9-driven homologous recombination. Transgenic parasites are selected for using drug treatment and propagated in immunodeficient mice

however, other models such as NOD-SCID-gamma (NSG) may also be suitable.

The success of this protocol depends heavily on the transfection efficiency achieved with *Cryptosporidium*. For this reason, we recommend optimization of transfection in vitro prior to attempting to make stable transgenics. Transfection of *Cryptosporidium* was first pioneered using a BTX electroporator (Harvard Apparatus, MA, USA), and then optimized using an AMAXA 4D-Nucleofector (Lonza, Basel, Switzerland). We have included the steps for transfection using the AMAXA 4D-Nucleofector, as this delivers superior efficiency (please *see* Pawlowic et al. [5] for detail on other instruments). To pilot transfection, we recommend using a simple *Cryptosporidium* Nanoluciferase expression vector [4]. The sensitivity of the Nanoluciferase assay allows for the detection of even low levels of transfection. The transfection efficiency of *Cryptosporidium* declines as the parasites age, so we recommend using oocysts that were recently isolated from infected animals, ideally within the past 4 months.

2 Materials

2.1 Optimizing Transfection with Transient Expression of Nanoluciferase

2.1.1 Excystation

1. 1×10^7 viable *Cryptosporidium parvum* oocysts.
2. Phosphate buffered saline (PBS), pH 7.4.
3. Household bleach, diluted 1:4 in deionized water.
4. Sodium taurodeoxycholate: 0.2 mM dissolved in PBS.
5. Water bath or incubator set to 37 °C.
6. Microcentrifuge.
7. 1.5 mL microcentrifuge tubes.
8. Hemocytometer.

2.1.2 Transfection

1. AMAXA 4D-Nucleofector (Lonza Group AG, Basel, Switzerland).

2. SF Cell Line 4D-Nucleofector X Kit S (Lonza).
3. 10 μg *Cryptosporidium* Nanoluciferase expression vector [4].

2.1.3 Cell Monolayer Infection

1. 24-well tissue culture plate with HCT-8 cells (ATCC) grown to 70% confluency.
2. Infection media: RPMI or DMEM with 1% fetal bovine serum.

2.1.4 Measuring Nanoluciferase

1. Nano-Glo Luciferase Assay System (Promega Corp., Fitchburg, WI).
2. White 96-well polystyrene plates (medium binding) for reading luminescence (Corning Inc., Corning, NY).
3. Plate reader capable of measuring luminescence.

2.2 Creation of Stable Transgenic *Cryptosporidium parvum*

2.2.1 Excystation

1. 2.5×10^7 viable *Cryptosporidium parvum* oocysts.
2. Phosphate buffered saline (PBS), pH 7.4.
3. Household bleach, diluted 1:4 in deionized water.
4. Sodium taurodeoxycholate: 0.2 mM dissolved in PBS.
5. Water bath or incubator set to 37 °C.
6. Microcentrifuge and 1.5 mL microcentrifuge tubes.
7. Hemocytometer.

2.2.2 Transfection

1. AMAXA 4D-Nucleofector (Lonza).
2. SF Cell Line 4D-Nucleofector X Kit L (Lonza).
3. 50 μg Cas9/gRNA expression vector [4] (*see* **Notes 1 & 2**).
4. 50 μg PCR segment for homologous repair (*see* **Notes 1 & 3**).

2.2.3 Mouse Infection

1. 1 mL syringes with needle tips for gavage.
2. Saturated sodium bicarbonate solution (8% w/v).
3. One cage of four adult IFNγ KO mice (B6.129S7-*Ifng*^{tm1Ts}/J)) (Jackson Labs, Bar Harbor, ME).
4. 16 mg/mL paromomycin sulfate salt dissolved in sterile water.
5. Optional: ampicillin, streptomycin, and vancomycin.

2.2.4 Collection

1. DietGel Boost, calorie supplement for mice (Clear H_2O, Portland, ME).
2. Dedicated forceps for fecal collection.
3. Optional: Raised wire mesh flooring for cage collection.

2.2.5 Purification

1. Tap water.
2. LabGen 125 homogenizer (Cole-Parmer, Vernon Hills, IL).

3. Saturated sucrose flotation solution: 1.33 specific gravity (756 g sucrose dissolved in 483 mL deionized water produces 1 L).
4. Saline solution: 0.85% NaCl in deionized water.
5. CsCl solution: 1.25 M (21.75 g dissolved in 103.25 mL deionized water).
6. 50 mL conical tubes.
7. 500 mL centrifuge tubes.
8. 2 mL low-binding microcentrifuge tubes.
9. 1.5 mL microcentrifuge tubes.
10. Tissue cell scrapers.
11. 250 μm wire mesh Micro Sieve Set (Bel-Art, Wayne, NJ).
12. Tabletop centrifuge.
13. Microcentrifuge.
14. Hemocytometer.

3 Methods

3.1 Optimizing Transfection with Transient Expression of Nanoluciferase

3.1.1 Excystation

To transfect *Cryptosporidium*, it is necessary to rupture the protective oocyst wall and release the motile sporozoites. We use the well-established combination of sodium taurodeoxycholate and elevation of temperature to 37 °C to induce excystation [6]. To test your electroporation efficiency, begin by transfecting 1×10^7 sporozoites with a simple Nanoluciferase expression vector. We recommend starting with 1×10^7 oocysts for this protocol because even with lower excystation rates, 50%, for example, sporozoite numbers should suffice to observe transfection: 1×10^7 oocysts × 0.5 excystation rate × 4 sporozoites per oocyst = 2×10^7 excysted sporozoites.

1. Resuspend 1×10^7 oocysts in 500 μL of cold diluted household bleach [1, 4]. Incubate on ice for 10 min. Oocysts are naturally resistant to chlorine treatment, and this incubation is intended to kill any bacterial contaminants. Bleach treatment also enhances wall permeability and can enhance excystation.
2. Centrifuge at 16,000 × *g* for 3 min, then resuspend in 1 mL cold PBS.
3. Repeat **step 2** to remove residual bleach.
4. Centrifuge at 16,000 × *g* for 3 min. Then, resuspend oocysts in 400 μL of 0.2 mM sodium taurodeoxycholate.
5. Incubate on ice for 10 min.
6. Add 1 mL PBS and incubate at 37 °C for 1 h.

7. Use a disposable cell counting chamber/hemocytometer to monitor excystation. Excystation rates vary depending on the viability of the oocysts, and we normally see excystation rates between 50% and 90%. Incubation at 37 °C can be prolonged up to 2 h to achieve a higher excystation rates if needed. If you are experiencing low excystation rates, consider making a fresh stock of sodium taurodeoxycholate.
8. Count sporozoites and aliquot 2 new 1.5 mL tubes with 1×10^7 each.

3.1.2 Transfection

As discussed in the introduction, we recommend optimizing transfection with a simple *Cryptosporidium* Nanoluciferase expression vector. The protocol below is specific to the AMAXA 4D-Nucleofector.

1. Resuspend 1×10^7 sporozoites in 15 μL complete SF buffer (12.3 μL Cell Line Solution + 2.7 μL Supplement 1).
2. Add 10 μg of *Cryptosporidium* Nanoluciferase expression vector resuspended in 5 μL of nuclease free water.
3. Transfer sporozoites and DNA into one well of the 16-well cuvette strip and electroporate using the program "EH100."
4. Move transfected sporozoites into a new 1.5 mL tube and keep on ice until infection.

3.1.3 Cell Monolayer Infection

1. Replace growth medium of a 70% confluent 24-well HCT-8 culture with infection medium.
2. Distribute sporozoites equally between three wells of the 24-well plate.
3. Incubate for 24–48 h at 37 °C.

3.1.4 Measuring Nanoluciferase

1. Combine Nanoglo substrate and lysis buffer at a 1:50 ratio.
2. Remove media from wells and replace with 500 μL substrate and lysis buffer.
3. Mix thoroughly with a 1 mL pipette.
4. Aliquot 200 μL to the 96-well luciferase plate. You should have enough to measure each well from the 24-well plate in duplicate.
5. Measure luminescence.

3.2 Establish Stable Transgenic *Cryptosporidium parvum* Parasites

3.2.1 Excystation

To create a stable transgenic strain of *Cryptosporidium* we typically infect a cage of four mice. Each mouse should be infected with 1×10^7 transfected sporozoites, therefore, we recommend starting with 2.5×10^7 oocysts. Again, even with a low excystation rate of 50%, you will still have enough sporozoites to attempt transfection (2.5×10^7 oocysts × 0.5 excystation rate × 4 sporozoites per oocyst = 5×10^7 excysted sporozoites).

1. Resuspend 2 × 10^7 oocysts in 500 µL of cold diluted household bleach. Incubate on ice for 10 min.
2. Centrifuge at 16,000 × *g* for 3 min, then resuspend in 1 mL cold PBS.
3. Repeat **step 2** to remove any residual bleach.
4. Centrifuge at 16,000 × *g* for 3 min, then resuspend oocysts in 400 µL sodium taurodeoxycholate.
5. Incubate on ice for 10 min.
6. Add 1 mL PBS and incubate a 37 °C for 1 h.
7. Use a hemocytometer to monitor excystation. Excystation rates vary depending on the viability of the oocysts, and we normally see excystation rates between 50 and 90%. Incubation at 37 °C can be prolonged up to 2 h to achieve a higher excystation percentage. If you are experiencing low excystation rates, consider making a fresh stock of sodium taurodeoxycholate.
8. Count sporozoites and aliquot 5 × 10^7 to a new 1.5 mL tube on ice.

3.2.2 Transfection

This protocol is specific to the AMAXA 4D-Nucleofector.

1. Resuspend sporozoites in 80 µL complete SF buffer (65.6 µL Cell Line Solution +14.4 µL Supplement 1).
2. Add 20 µL of concentrated DNA (50 µg Cas9/gRNA expression plasmid +50 µg PCR segment for homologous repair).
3. Transfer to cuvette and electroporate using the program "EH100."
4. Transfer to a new 1.5 mL tube and add 300 µL cold PBS. Keep transfected sporozoites on ice until infection.

3.2.3 Mouse Infection

Once out of the protective oocyst, sporozoites lose their ability to survive the harsh environment and acidity of the host stomach. The most effective way to bypass this problem is to surgically inject transfected sporozoites directly into the small intestine, a method explained in detail here [5]. However, sporozoites can also be successfully delivered to the small intestine by simpler oral gavage if the stomach acid is neutralized with sodium bicarbonate prior to infection.

Optional: Pretreat mice with antibiotics prior to infection. In our hands, pretreatment with an antibiotic cocktail for 1 week increases Cryptosporidium parvum *infection. Mice are given ampicillin (1 g/L), streptomycin (1 g/L), and vancomycin (0.5 g/L)* ad libitum *in their drinking water for 1 week. Mice are then provided normal drinking water upon infection.*

1. Gavage each mouse with 200 μL of saturated sodium bicarbonate and wait 5 min.
2. Gavage each mouse with 100 μL of transfected sporozoites.
3. After allowing the sporozoites to infect overnight, change the cage drinking water to start in vivo selection with 16 mg/mL paromomycin. We recommend that mice be treated with paromomycin for no longer than 4 weeks to avoid toxicity from chronic exposure.

3.2.4 Collection

Mice will typically begin to shed transgenic parasites between the 7th and 14th day post infection. Parasite shedding can be monitored from collected fecal samples by qPCR using a *Cryptosporidium*-specific set of probe and primers [7]. More conveniently, the Nanoluciferase reporter component of our standard selection cassette can be monitored in collected fecal samples without need for DNA isolation, thus greatly reducing the workload associated with establishing parasite shedding from multiple cages. Mice should be monitored daily for signs of distress, including hunching, lethargy, and significant weight loss. To mitigate weight loss during infection, we supplement mouse diet with DietGel Boost at the onset of parasite shedding.

Once mice begin to shed transgenic parasites, start daily fecal collections. Take extra precautions to avoid cross-contamination of transgenic strains. Each strain should have its own collection cage and forceps, and mouse handlers should change gloves before and after handling infected mouse cages. Moving mice to cages with wire mesh floors during the day is a simple way to maximize collections. Store all collected fecal samples at 4 °C until purification and avoid freezing.

3.2.5 Purification

To purify oocysts, we use sucrose flotation followed by CsCl gradient centrifugation. There are many other purification methods for *Cryptosporidium* oocysts, including sucrose gradients, Nycodenz gradients, ether treatment, and sodium chloride flotation, and you may want to optimize this for your specific requirements [8]. Keep in mind the need to balance oocyst yield, purity, and viability. To maximize viability, all purifications should be done on ice with cold reagents.

1. Gather collected fecal samples (approximately 5–10 days of collection per purification) into a 50 mL conical tube and add 35 mL cold tap water.
2. Thoroughly blend samples. For this, we recommend using a handheld homogenizer, but this can be done manually.
3. Filter samples through a 250 μm wire mesh and into a 500 mL centrifuge tube. Pour 450 mL cold water over the sample and stir with a cell scraper to dislodge oocysts.

4. Centrifuge at 1000 × *g* for 10 min at 4 °C.
5. Resuspend pellet in 50 mL cold tap water and split into two 50 mL conical tubes.
6. Add 25 mL (1:1) cold saturated sucrose solution to conical tubes and invert several times to mix.
7. Centrifuge at 1000 × *g* for 5 min at 4 °C.
8. Carefully decant supernatant into a new 500 mL centrifuge tube and add 450 mL cold tap water.
9. Centrifuge at 1500 × *g* for 15 min at 4 °C.
10. Resuspend pellet in 5 mL cold saline solution.
11. Slowly overlay 1 mL of sample onto 1 mL of cold CsCl solution. For this, we use low-binding 2 mL centrifuge tubes.
12. Centrifuge at 16,000 × *g* for 3 min at 4 °C.
13. Oocysts should be visible as a white layer at the saline–CsCl interface. Slowly pipette 0.75 mL from the interface and transfer into a new 1.5 mL tube. Add 0.75 mL saline solution.
14. Centrifuge at 16,000 × *g* for 3 min at 4 °C.
15. Discard supernatant and combine pellets from all tubes using 1 mL cold saline solution.
16. Count oocysts using a hemocytometer to determine yield.

4 Notes

1. *Concentration of DNA for transfection:* The protocols for transient and stable transfection call for highly concentrated DNA. We recommend using ethanol precipitation to concentrate plasmid DNA and/or PCR products prior to transfection.
2. *Design of Cas9 guide RNA:* There are a number of resources to help design an appropriate guide RNA. Eukaryotic Pathogen CRISPR guide RNA/DNA Design Tool (EuPaGDT) has the genome of *Cryptosporidium parvum* preloaded and allows for adjustable on-target and off-target search parameters [9]. For a walk-through of cloning guide RNA into a *Cryptosporidium* Cas9/U6 vector, *see* ref. 5. Keep in mind that recombination efficiency increases when the Cas9 cut site is located in close proximity to either of the homologous flanks.
3. *Design of PCR segments for homologous repair:* The amount of homologous DNA required for recombination in *Cryptosporidium* is surprisingly low, around 50 bp. With this low requirement, you can create homologous regions simply by adding them to the 3′ end of primers used to amplify the selection cassette (Fig. 2). When designing your selection

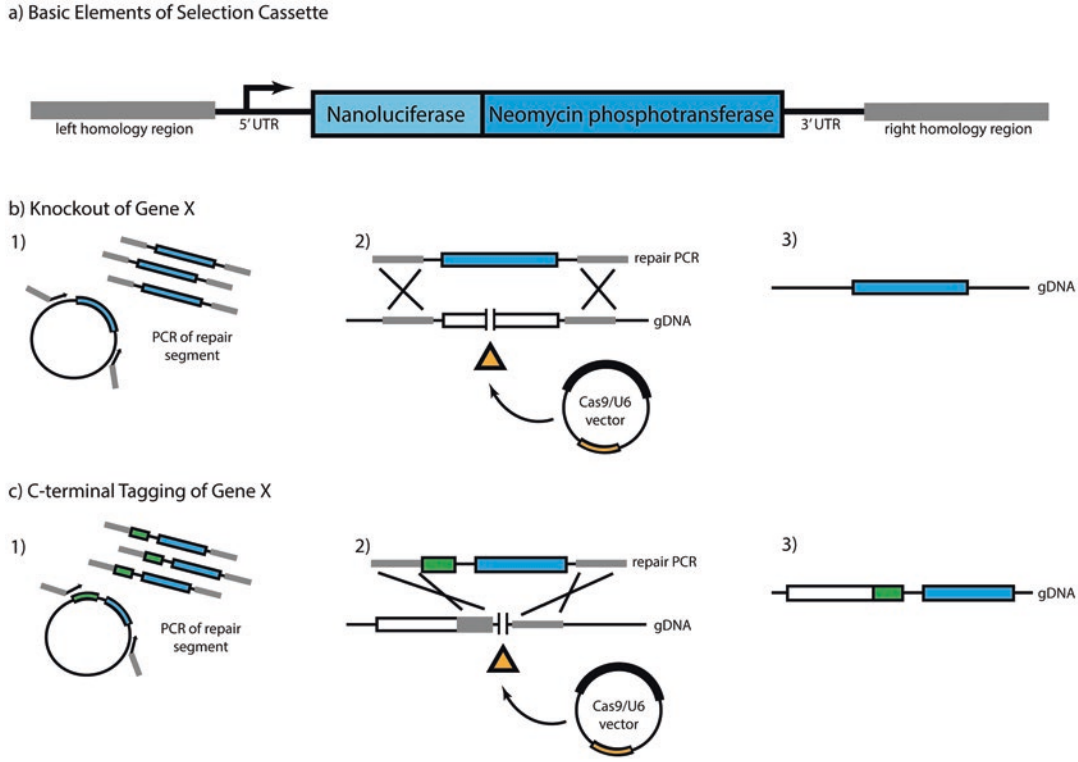

Fig. 2 Designing a selection cassette for *Cryptosporidium parvum.* (**a**) The basic elements of a selection cassette are a reporter (Nanoluciferase) and selection marker (Neomycin phosphotransferase). To ensure continuous expression, these genes should be flanked by 5′ UTR and 3′ UTR DNA from a constitutively active *Cryptosporidium* gene (e.g., actin). (**b**) To target a gene for KO, design your Cas9 guide RNA to target a sequence within your gene of interest. Then, amplify your selection cassette with PCR primers containing homologous regions flanking this gene of interest. (**c**) To C-terminally tag a gene of interest, first you will need a tagging sequence (e.g., HA, FLAG, MYC) and 3′ UTR upstream of your selection cassette. When designing your left homology primer to amplify this tagging cassette, remember to make sure that your gene is in frame with the coding sequence of this new tag. Next, design your Cas9 guide RNA to target an area in the 3′ UTR of your gene of interest. Your right homology primer can then be designed using genomic DNA located 3′ to this Cas9 recognition site. Pay close attention to the proximity of nearby genes because *Cryptosporidium* has a very compact genome

cassette, remember that you must include neomycin phosphotransferase, which provides resistance to paromomycin. Your parasites will be constantly exposed to paromomycin during mouse infection, so drive the expression of this resistance marker with a constitutive *Cryptosporidium* promoter. We have successfully used the promoters for actin, tubulin, enolase, and aldolase for constitutive expression. Adding a luminescent reporter, such as Nanoluciferase, to your selection cassette will make it easier and faster to measure shedding of transgenic parasites. We commonly use a translational fusion of these two genes for our selection cassettes.

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